

ELECTROPHORETIC ANALYSES OF HEMOLYMPH PROTEINS
OF PTERONARCYS CALIFORNICA

by

ROBERT E. STEVENSON

A thesis submitted to the Graduate Faculty in partial
fulfillment of the requirements for the degree

of

MASTER OF SCIENCE

in

Zoology

Approved:

Head, Major Department

Chairman, Examining Committee

Dean, Graduate Division

MONTANA STATE UNIVERSITY
Bozeman, Montana

August, 1966

VITA

Robert Eugene Stevenson was born on July 17, 1937 in Great Falls, Montana, the son of Mr. and Mrs. W. W. Stevenson.

He completed high school in May of 1955 and graduated from the College of Great Falls with a Bachelor of Arts degree in biology in June of 1962.

He entered Montana State University in September of 1964 and received a NIH graduate research assistantship in April of 1965. He married Pauline Hodgson of Lakeland, Florida in August of 1965 and completed his Master of Science degree in zoology in August, 1966.

ACKNOWLEDGEMENTS

Sincere thanks are due Dr. C. J. D. Brown who directed the study and aided in the preparation of the manuscript.

Mr. Robert V. Thurston and Dr. Gary A. Strobel gave technical advice and encouragement. David Gillespie and Richard Vincent helped with the identification of stoneflies.

Financial support was provided by Grant WP-00438-01A2 from the Division of Water Supply and Pollution Control, Public Health Service.

TABLE OF CONTENTS

	PAGE
LIST OF TABLES	v
LIST OF FIGURES	vi
ABSTRACT	vii
INTRODUCTION	1
METHODS	2
Collection, Hemolymph Removal and Electrophoretic Procedures . .	2
Stress	3
Analyses	4
RESULTS	5
<u>Pteronarcys californica</u>	6
Protein Pattern Variations in Hemolymph of Males and	
Females	8
Pattern Variations Among Nymphs from Different Streams	8
Pattern Variations Resulting from Stress	9
DISCUSSION	14
LITERATURE CITED	15

LIST OF TABLES

Table	PAGE
I. Means and ranges (percent) of hemolymph protein concentrations taken from nymphs of <u>P. californica</u>	7
II. Band arrangement differences among nymphs	9
III. Means and ranges (percent) of hemolymph protein concentrations taken from stressed nymphs of <u>P. californica</u>	13

LIST OF FIGURES

Figure	PAGE
1. Electrophoretic protein patterns of hemolymph from stonefly nymphs	11
2. Means of hemolymph protein concentrations from nymphs taken in different rivers	12

ABSTRACT

Electrophoretic protein patterns of hemolymph were determined for 207 stonefly nymphs from Southwestern Montana between June 1965 and April 1966. Of these, 194 were Pteronarcys californica. Distinct differences were found among the patterns obtained from Pteronarcys, Pteronarcella, Acroneuria and Arcynopteryx. P. californica had a characteristic protein pattern although intraspecific differences were observed. No differences were found between the patterns of males and females. Significant differences in protein concentration for certain bands were found among nymphs of P. californica taken from the West Gallatin River, Madison River and Yellowstone River. Nymphs subjected to light, dehydration and various concentrations of CuSO_4 were found to have significantly different concentrations of protein in several bands.

INTRODUCTION

The hemolymph of certain stoneflies from Montana was studied electrophoretically during the period June 1965-April 1966. The objectives were to: make an intensive study of Pteronarcys californica from one area; determine if there were intergeneric differences in hemolymph composition between P. californica and other stoneflies; determine if stress alters the hemolymph pattern of P. californica; compare the patterns of P. californica from several areas. P. californica was selected for use in this study because of its large size and availability.

The blood of a variety of animals has been subjected to electrophoretic analyses. Individual variations were found among the larvae of Eacles imperialis and starvation affected the hemolymph protein pattern of Malacosoma americanum larvae (Whittaker and West, 1962). Specific electrophoretic patterns were found in the arachnid families of Ixodidae and Argosidae (van Sande and Karcher, 1960). Electrophoretic patterns of fish exposed to various pollutants were noticeably different from those of unexposed fish (Fujiya, 1961). Electrophoretic differentiation of Anolis lizard populations was accomplished by Gorman and Dessauer (1965).

The stoneflies used in my study were collected in the following places: West Gallatin River near Axtell's bridge, Gallatin County; Madison River near the bridge on Highway 289, Madison County; Yellowstone River near Carter's bridge, Park County.

METHODS

Collection, Hemolymph Removal and Electrophoretic Procedures

Stonefly nymphs were collected with a wire screen and 10-12 specimens were then placed in a gallon jar filled with river water and transported to the laboratory. The time between collection and hemolymph removal was usually 2 hr. Heparinized microhematocrit tubes (75 mm) were used to remove the hemolymph. These were prepared by drawing out one end over a flame and then breaking it off to the desired opening size. The tip was inserted into a nymph at approximately a 30° angle to the horizontal immediately posterior to the metathoracic tergite. Tubes containing hemolymph were centrifuged for 5 min. at 15,500 RCF. The sediment was then discarded and the supernatant placed in the refrigerator at 3-5 C. Lowry's method (1951) was used for protein determinations. Approximately 200 μ g of protein was analyzed in each gel. Electrophoresis was carried out according to the method of Ornstein and Davis (1962) with equipment modifications described by Thurston (1966). The large pore spacer gel was omitted because of polymerization difficulties. The protein sample was mixed with 0.15 ml of large pore gel, layered on the small pore gel column, and left in fluorescent light for 20-25 min. Gels were inserted in the upper of the two buffer reservoirs and buffer was carefully added to avoid disturbing the large pore gel. This procedure was followed regardless of polymerization in the large pore-sample gel. Eight gels were run for 1 hr. with a total of 24 ma being applied to the system. The small pore gel was prepared at room temperature whereas the large pore gel and electrophoresis were done at 5° C.

Stress

Nymphs of P. californica were subjected to drying and to light exposure to determine if these treatments alter the electrophoretic protein pattern. Ten nymphs were placed in a jar without water but containing damp paper towels and kept in a refrigerator (5 C) for 6 days. Comparisons were made between their electrophoretic patterns and those of freshly collected nymphs from the West Gallatin River. Ten newly-caught nymphs were kept in a jar containing 5 liters of continuously aerated water (11 C). These were exposed to a 100 watt bulb suspended 12 inches from the side of the jar for 6 days. The electrophoretic patterns of the light-exposed nymphs were compared with controls taken from the West Gallatin River.

Nymphs were subjected to various concentrations of CuSO_4 to see if chemical stress altered the electrophoretic pattern. Copper sulfate was used in the following concentrations: 0.4, 0.8, 10.0 and 50.0 mg/liter. Five liters of dechlorinated tap water were used in each jar, and rocks were added to provide concealment and footing. Water temperatures ranged from 4 to 15 C (average 8 C). Solutions were replaced daily and the jars were rinsed between changes. Pattern comparisons were made between the 0.4 and 0.8 mg/liter groups and a control group whereas the 1.0 and 10.0 mg/liter groups were compared with nymphs taken directly from the West Gallatin River. No comparisons were made with the 50 mg/liter group because the volume of hemolymph extracted was insufficient for centrifugation or to run a Lowry's test. Chemical characteristics of water used

during the test were: dissolved oxygen 9.2-10.4 ppm, pH 7.3-8.3, phenolphthalein alkalinity 0 ppm, methyl orange alkalinity 90-91 ppm and total hardness 90-123 ppm.

Analyses

A Joyce Chromoscan recording densitometer was used to obtain a graphic profile of the gels. Absorption of light by Amido Schwarz dye does not follow a linear relationship therefore a specially designed cam was installed in the Chromoscan so the height of the curve at any point is in direct linear relationship to total stained protein present. A polar planimeter was used to determine the total area of all bands as recorded by the densitometer and that of the individual bands from the profile. A percentage was then determined by dividing individual areas by the total area. Visual comparisons were made by placing gels against a light colored background and noting differences in band arrangement. A nomogram was used to determine changes in distances migrated by the various bands. The gels in which the lead band migrated the furthest and least distances were used to construct the nomogram. Lines were drawn on paper connecting identical bands of the two gels. Distances migrated by the bands of other gels were compared with those on the nomogram and variations in the distances migrated could then be quickly detected. One way analysis of variance and Duncan's New Multiple Range test were used in the statistical analyses of data.

RESULTS

The electrophoretic protein patterns of 207 stonefly nymphs were determined and of these 194 were Pteronarcys californica. The others included the following genera: Arcynopteryx, Acroneuria and Pteronarcella. The number of bands, approximate distance migrated by the lead band (no. 1) and the band arrangement were used for all comparisons. The number of bands found in each genus and the distance migrated by band no. 1 are as follows:

Genus	No. Bands	Distance (mm) Migrated
		Band no. 1
<u>Acroneuria</u>	15	28
<u>Arcynopteryx</u>	13	33
<u>Pteronarcella</u>	10	31
<u>Pteronarcys</u>	13	34

Distinct differences were found in the arrangement of bands with respect to each other and to the large pore gel (Fig. 1A). Pteronarcella was distinguished by the widest separation (17 mm) between bands no. 1 and 4 (bands no. 2 and 3 were barely discernible). Acroneuria was the only one with 7 heavily stained bands while the others had five or less. Arcynopteryx was distinguished from the others by having 6 moderately stained and one heavily stained band within 22 mm of the origin. Pteronarcys was distinguished from the others in having the first 4 bands separated by 13 mm with nearly equal spaces between them. Three to 5 μ liters of hemolymph were used to obtain the electrophoretic patterns of Acroneuria, Arcynopteryx and Pteronarcella. Lowery's test was not carried out on their hemolymph so the gels were not analyzed on the Chromoscan.

Pteronarcys californica

Hemolymph of nymphs taken from one locality in the West Gallatin River was analyzed electrophoretically to determine pattern variations. Ten males (29-42 mm) and 17 females (32-55 mm) taken between July 27, 1965 and January 11, 1966 were used. The distance migrated by the bands, band arrangement and the relative amount of protein in each band was used to evaluate differences. The average distances (mm) migrated from the large pore gel for each band were as follows: 1-34, 2-31, 3-27, 4-22, 5-16, 6-11, 7-8, 8-6, 9-3. The number of bands between band no. 9 and the large pore gel varied from 0 to 4. These were found in most gels. The greatest width of these barely discernible bands was 0.1 mm and the distance migrated in most experiments were from 1 to 3 mm. Bands no. 8 and 9 migrated 2 mm further in one gel than in any of the others. Only five of the 27 gels were satisfactory for Chromoscan analyses. The most common variations were in bands no. 2, 4, 6 and 9 (Fig. 1B). Band no. 2 was absent in 60% of the gels and 20% of bands no. 4 and 6 were each split into two bands. In 4 of 5 gels band no. 9 showed partial separation into two bands however complete separation might have occurred with longer running time. No two nymphs had identical concentrations of protein in all cases. Of those measured the least amount of protein was found in band no. 2 and the most in band no. 6. The others, in increasing order of protein concentration were 1 and 4, 3, 5, 8, and 7. The protein density of bands 9-13 was not determined. Band no. 9 was omitted because the protein concentration was consistently greater than could be measured by the

Protein Pattern Variations in Hemolymph of Males and Females. The electrophoretic patterns of 21 females and 14 males from all streams were compared visually. Ten females and 7 males from one lot were compared using the Chromoscan. Visual and nomographic examinations of gels revealed no differences attributable to sex. There were no marked differences in mean protein concentration per band for 3 males (33-44 mm) and 3 females (52-54 mm) taken the same day in the Madison River. Females had 1.9% greater mean protein concentration in band no. 1 than males, but males had 1.3% greater protein concentration than females in band no. 7. These small differences are probably due to intraspecific differences not related to sex.

Pattern Variations Among Nymphs from Different Streams

Nymphs were secured from the West Gallatin River, Madison River and the Yellowstone River between November 17, 1965 and January 4, 1966 for comparison of electrophoretic patterns. Nomographic analyses of electrophoretic protein patterns did not show differences in relative distances migrated by identical bands. Visual comparisons of the gels revealed differences in the arrangement of bands no. 2, 4, 6 and 9. These were either absent, split into two bands or partially split (Table II, Fig. 1C).

Protein concentration in identical bands were statistically compared using one way analysis of variance. Significant differences at the 5% level were found between nymphs from the three streams. Nymphs from the West Gallatin River and Madison River had significantly different protein

concentrations in bands no. 1, 6, 7 and 8; those from the West Gallatin River and Yellowstone River in bands no. 4, 6 and 7 and those from the Yellowstone River and Madison River in bands no. 4, 6, 7 and 8. The mean protein concentration per band for nymphs taken in the three streams were compared (Fig. II, Table I). The greatest variation in mean protein concentration was found in band no. 6 (35.5% to 50.9%), the least in band no. 5 (6.5% to 6.9%)

Table II. Band arrangement differences among nymphs.

Stream	No. Specimens	Band	Band No.			
			2	4	6	9
West Gallatin	5	absent	3			
		partially split				3
		split		1	1	
Madison	6	absent	1			
		partially split				6
		split		2	5	
Yellowstone	5	absent				
		partially split				3
		split		3	5	

Pattern Variations Resulting from Stress

Sixty-five nymphs collected between February 7 and April 29, 1966 in the West Gallatin River were used to test the effects of various stresses. Stress did not cause detectable changes in the number of bands or their arrangement. Stressed individuals showed no more variations in partial splitting, complete splitting or presence of bands than did controls (Fig.

1D). Nomogram analyses of hemolymph patterns obtained for 6 nymphs subjected to 50 mg/liter CuSO_4 showed that bands no. 7, 8 and 9 migrated 2-8 mm further in the gels of 4 of these specimens. However, this hemolymph had not been centrifuged (resulting in non-removal of heavy particles) and this rather than stress may account for the difference in migration. Six nymphs were subjected to 0.4 mg/liter CuSO_4 and band no. 9 in gels from all of these migrated past the nomogram limit for distances ranging from 1-3 mm. This band also migrated 1 mm past the limit in one control. The reason for this is obscure.

One way analysis of variance showed significantly different protein concentrations in some bands at the 5% level. The ranges and means in protein concentration per band resulting from stress treatment are shown in Table III. Significant differences were found for the following concentrations of CuSO_4 : 0.4 mg/liter group in band no. 8; 0.8 mg/liter group in band no. 2; 1 mg/liter group in bands no. 1, 6 and 8; 10 mg/liter group in bands no. 1, 6 and 7. Hemolymph protein concentrations of nymphs subjected to dehydration and light exposure were compared with controls and significant differences were found in bands no. 2, 4 and 6. The mean volume of hemolymph required to obtain 200 μg protein was less in stress groups (2.18 μliters) than in nymphs taken from the three rivers (2.92 μliters).

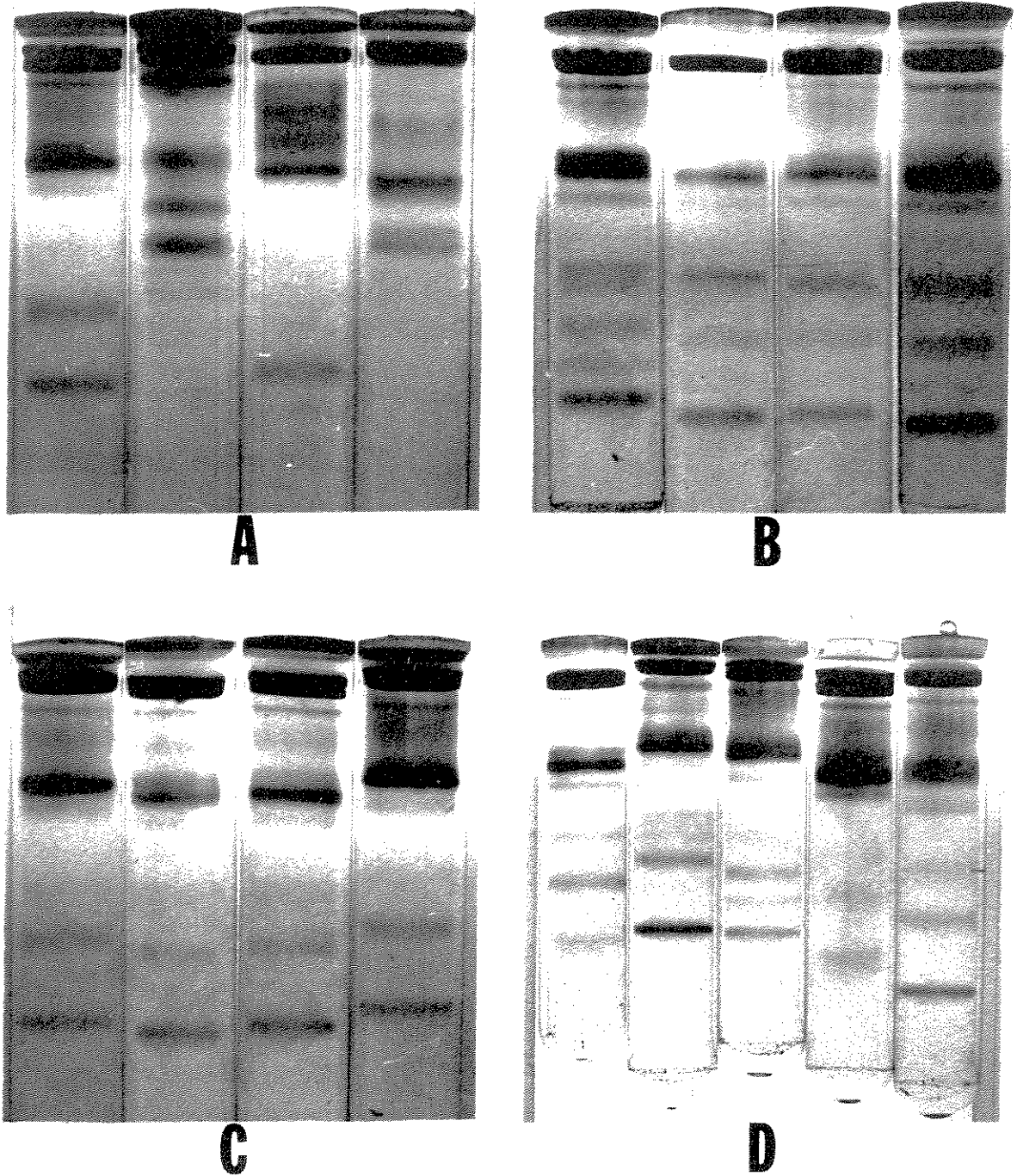


Figure 1. Electrophoretic protein patterns of hemolymph from stonefly nymphs. A. Intergeneric differences (left to right) *Pteronarcys*, *Arcynopteryx*, *Pteronarcella*, *Acroneuria*. B. Intraspecific differences in *P. californica*. C. Pattern variations between nymphs from the West Gallatin River (outside gels) and Madison River (inside gels). D. Patterns from nymphs subjected to stress. Left to right: unstressed, dehydration, 1 mg/liter CuSO_4 , 10 mg/liter CuSO_4 , light.

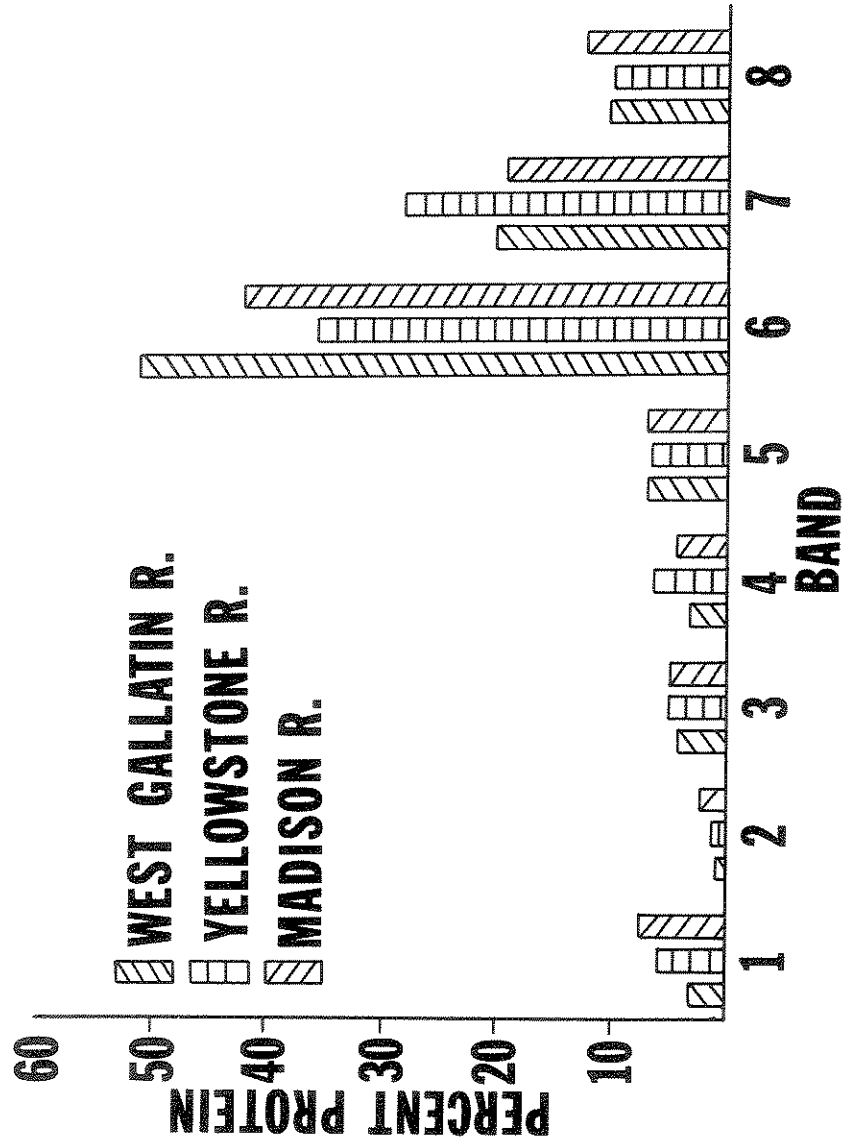


Figure 2. Means of hemolymph protein concentrations from nymphs taken in different rivers.

Table III. Means and ranges (percent) of hemolymph protein concentrations taken from stressed nymphs of P. californica.

Type of stress	No. nymphs	Band No.							
		1	2	3	4	5	6	7	8
Copper Sulfate 10 mg/l	4	6.7	2.6	5.1	3.5	4.6	54.4	14.0	9.3
		2.9-11.5	2.8-7.5	4.0-6.2	1.2-5.7	3.8-5.5	51.8-55.7	11.4-18.7	7.9-10.4
1 mg/l	7	6.5	1.3	4.4	4.0	5.0	41.7	22.2	15.2
		3.9-9.2	0-3.6	2.6-6.1	2.3-5.3	2.6-7.6	37.1-44.6	15.9-29.8	11.4-25.0
0.8 mg/l	6	3.8	1.8	2.3	1.9	9.8	34.5	31.0	14.7
		2.3-5.8	0-4.1	0-3.9	0-4.5	3.6-22.9	13.4-47.9	14.1-58.8	12.8-17.8
0.4 mg/l	5	3.7	1.3	3.3	3.6	7.0	49.9	20.3	10.6
		2.3-6.7	0-4.3	1.6-4.3	2.3-5.5	3.7-9.7	41.3-57.0	10.8-22.5	5.7-12.2
Control	5	9.1	1.0	5.5	4.2	13.5	36.2	17.7	12.5
		3.8-15.7	0-4.0	2.9-9.8	1-9.8	7.5-22.4	14.7-46.0	13.7-22.6	10.6-13.8
Light	4	7.6	2.0	6.1	4.9	8.3	38.5	20.6	11.9
		6.1-10.6	0-3.6	3.1-9.1	4.3-5.8	7.0-9.4	32.6-42.6	14.5-25.4	10.9-13.3
Dehydration	7	7.7	1.1	6.1	6.3	7.4	42.0	17.7	11.3
		4.9-11.3	0-3.3	3.4-8.0	4.3-9.3	5.2-9.4	24.1-47.6	13.2-31.2	9.4-14.4

DISCUSSION

One advantage of disc- over paper- electrophoresis is in the greater number of protein fractions which can be distinguished. With its high resolving power, disc electrophoresis may be more useful in separating genera of stoneflies because slight differences in their hemolymph proteins are readily revealed. Numerous variations were found in the electrophoretic protein patterns of the relatively few Pteronarcys californica studied, and it is likely that the range of variations is greater than that found for this species. The number of specimens used for the statistical analyses in the stress experiments and stream difference comparisons was quite small. This makes statistical interpretation less valid. The use of disc electrophoresis as a tool in detecting changes in hemolymph protein of nymphs subjected to stress may be a useful tool in pollution studies.

LITERATURE CITED

- Fujiya, Masaru. 1961. Use of electrophoretic serum separation in fish studies. *Journal Water Pollution Control Federation*, 33(3): 250-257.
- Gaufin, Richard F. and Arden R. Gaufin. 1961. The effect of low oxygen concentrations on stoneflies. *Proceedings Utah Academy Sciences, Arts, Letters*, 38: 57-64.
- Gorman, George C. and Herbert C. Dessauer. 1965. Hemoglobin and transferrin electrophoresis and relationships of island populations of *Anolis* lizards. *Science*, 150: 1454.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and Rose J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- Ornstein, L. and B. J. Davis. 1962. Disc electrophoresis. *Distillation Products Industries*. Eastman Kodak Co. 62 p.
- Sande, Marc van and D. Karcher. 1960. Species differentiation of insects by hemolymph electrophoresis. *Science*, 131: 1103.
- Thurston, Robert V. 1966. Electrophoretic patterns of blood serum proteins from rainbow trout (*Salmo gairdneri*). Doctoral thesis, Montana State University.
- Whittaker, J. R. and A. S. West. 1962. A starch gel electrophoretic study of insect hemolymph proteins. *Can. J. Zool.*, 40: 655-671.